# LIGNIN STRUCTURE: RECENT DEVELOPMENTS

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**Abstract**

Studies of lignin structure and reactivity have been re-energized by the emergence of modern technologies, new analytical methods, and by exciting recent findings. The structural aspects which were considered complex but well understood some years back have seen revisions that add to the complexity in some ways but are clarified by the logical chemistry in others.

One of the most significant recent findings has been the elucidation of dibenzodioxocins as significant components of natural lignins. Now found in lignins from all classes of plants which have a guaiacyl lignin component, they are new structures derived from 5—5-coupling of lignin oligomers followed by coupling with a new lignin monomer; other lignification reactions then follow to fully incorporate these structures into the polymer. Another new structural unit, aryl-isochromans, expose a logical new pathway following β—1-coupling.

These new structures and others have been revealed by modern analytical and instrumental methods, particularly NMR. The now common use of 2D and 3D NMR, using pulsed field gradients, inverse detection, and modern digital instruments allows ever more sensitive detection and, more importantly, diagnostic interpretation. Because it is now possible to reveal a given unit in the complex polymer in a diagnostic way, structures are being suggested directly from spectra of the polymer (although confirmation and authentication using model compounds remains crucial).

Studying lignins from non-woody plants has also provided significant new knowledge. Acylated (e.g. acetylated, *p*-coumaroylated, *p*-hydroxybenzoylated) lignins are now better understood thanks to NMR methods, as well as new and established degradative methods (e.g. the DFRC method, and thioacidolysis) combined with mass-spectrometry. Acylated units can be a significant component of lignins; for example, lignins in the bast fibers of kenaf are more than 50% acetylated. Cell wall cross-linking in grasses by ferulates has also recently been clarified.

Mutant and transgenic plants in which steps in the monolignol biosynthetic pathway are down- or up-regulated are also providing a rich source of insight into the processes of lignification. Massive compositional shifts, significantly beyond traditional bounds, are found in a variety of mutants/transgenics. In some cases, units that are minor in normal lignins become major components. This facilitates their identification and may elucidate their radical-coupling reactions. Pathways interacting with lignification become identified, and the very definition of lignin itself becomes clouded or simplified depending on ones perspective.

This paper explores recent advances in understanding lignin structure, and provides applications that show the breadth and plasticity of the lignification process.

## Introduction

Lignification is the polymerization process in plant cell walls that takes phenolic monomers, produces radicals, and couples them with other monomer radicals (only during initiation reactions), or more typically cross-couples them with the growing lignin polymer/oligomer, to build up a phenylpropanoid polymer (Harkin, 1967; Freudenberg and Neish, 1968; Harkin, 1973; Brunow, 1998). As addressed below, there is current controversy over just what constitutes a lignin monomer or a lignin polymer. In the purest sense, lignins arise from radical coupling reactions of three primary precursors, the monolignols *p*-coumaryl, coniferyl and sinapyl alcohols, Figure 1. Since this process produces a polydisperse polymer with no extended sequences of regularly repeating units, its composition is generally characterized by the relative abundance of *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units (derived from each of the 3 primary monolignols) and by the distribution of interunit linkages in the polymer (e.g. β-aryl ether or β—O—4, phenylcoumaran or β—5, resinol or β–β biphenyl or 5—5, diphenyl ether or 4—O—5). There are also hydroxycinnamyl alcohol endgroups from the few initial dimerization reactions, structures such as non-cyclic α-aryl ethers (which arise from addition of a phenol to an intermediate β-aryl ether quinone methide) and various other structures.

***Often-overlooked Concepts; Differences Between Lignin and Lignans***

Lignans are dimeric products from radical coupling of monolignols. As such, they have structures analogous to their counterparts in the lignin polymer. However, comparisons of the relative amounts of linkages that arise during dimerization and those that occur during lignification overlooks the fact that dimerization is minimal in lignification where fewer products are possible, as depicted in Figure 1. A simple illustration should convince us that dimerization reactions are not terribly significant when looking at lignin structure. For example, in a softwood there are two possible outcomes of coniferyl alcohol dimerization reactions that can be approximately quantified. Either the dimer is a β–β-dimer, i.e. pinoresinol, or the dimer bears a cinnamyl alcohol sidechain that must remain following further lignification. The total amount of dimerization reactions can therefore be measured from the sum of the resinol products and the unsaturated sidechains in lignins. Estimates in softwoods for resinol structures are low, typically less than 2% (Adler, 1977; Chen, 1998). Unsaturated sidechains run typically less than 1%. Thus, monomer-monomer coupling accounts for considerably less than 5% of lignification in typical softwoods. Hardwoods have higher amounts, but still only ~3% (Adler, 1977), of β–β-coupled units (resinols), mainly syringaresinol units from sinapyl alcohol, but barely detectable levels of cinnamyl alcohol end-units. Again therefore, monomer-monomer coupling accounts for considerably less than 5% of lignification in typical hardwoods. Grasses and legumes are similar to hardwoods in this regard.

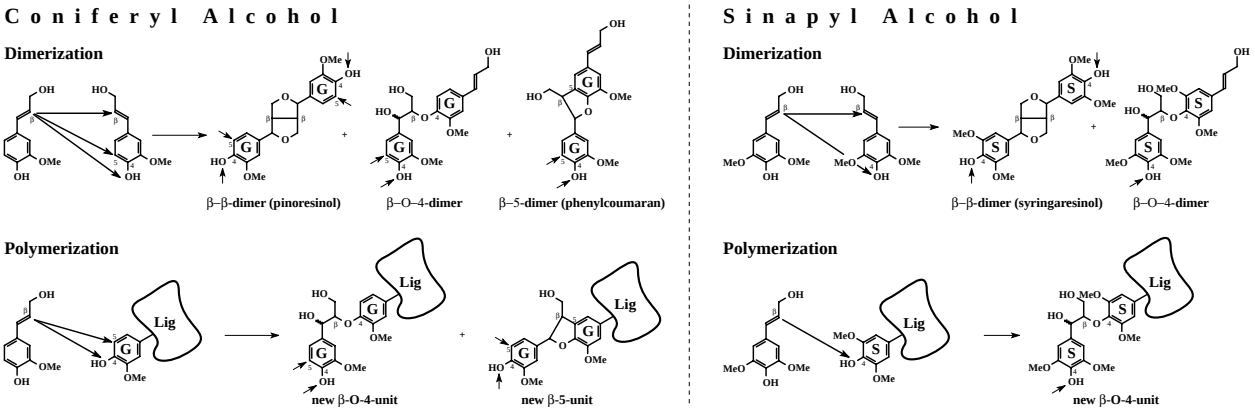


**Fig. 1. Radical coupling of monolignols, and differences between dimerization and lignin polymerization.** During lignification, dimerization reactions are rare the major linkages come from coupling of a monolignol radical with a radical of the growing lignin oligomer/polymer.

***Lignins are Optically Inactive***

Lignans, monolignol dimers, are often optically active. That is, they are produced by reactions in which the stereochemistry of the product is strictly controlled. Lewis group have recently documented a fascinating protein that specifically facilitates the coupling of two coniferyl alcohol radicals to produce a single optical isomer of pinoresinol (Davin *et al*., 1997). Such a protein is a significant finding in lignan chemistry/biochemistry. The group have further boldly extrapolated their findings to the process of lignification, suggesting that lignins also must be formed by carefully controlled processes (Lewis and Davin, 1998). However, there is currently no evidence for such a view. Lignins have always been considered optically inactive. Accommodating a lack of optical activity in lignins by the dirigent protein array theory of carefully controlled coupling unfortunately requires convoluted arguments (Lewis and Davin, 1998) that bring considerable conceptual difficulties, at least to this author (Ralph *et al*., 1999c; Sederoff *et al*., 1999). We and other groups have recently provided more significant evidence that lignins are indeed optically inactive. Matsumoto *et al*. examined ozonolysis products to demonstrate that β-aryl ethers are not optically active (Matsumoto *et al*., 1999). We showed that phenylcoumarans and resinols weren t either, and that β-aryl ethers likely weren t (Ralph et al., 1999c). These data complement early findings that resinol products from lignins had no detectable optical activity. Finally, we showed that lignins, isolated by means that could not affect the radically coupled centers, showed no detectable optical activity (Ralph et al., 1999c).

**New Analytical Method: The DFRC Method**

There are many good analytical methods for analysis of the composition and structure of lignins, but lignins are enormously complex. No one method can answer all questions. New methods continue to be developed and established methods continue to evolve. We recently developed an efficient approach to cleaving β-ether units in lignins (Lu and Ralph, 1997b; Lu and Ralph, 1997a; Lu and Ralph, 1998b) a method which differed from other established methods such as the acidolytic methods (e.g. acidolysis, thioacidolysis) and high-temperature base. Due to unique features of the method, it has provided new insights into structural features.

The method has been dubbed the DFRC method as an acronym for the chemistry involved (Derivatization Followed by Reductive Cleavage) and because it was developed at the US Dairy Forage Research Center (for which DFRC is also the acronym). As seen in simplified form in Figure 2, the method is firstly based on the solubilization of cell wall material by acetyl bromide. Under sufficiently mild conditions, AcBr cleanly acetylates alcohols and phenols, and brominates the benzylic positions of β-aryl ethers (Lu and Ralph, 1996). The key feature then is the cleavage of the resulting β-bromo-ether by (formally) two-electron reducing reagents, in this case zinc in acetic acid. The two reactions effect the cleavage of etherified and free-phenolic β-ethers and proceed in very high yield on model compounds. On lignin, cleavage of β-ethers results in the liberation of monomers, dimers and higher oligomers; the monomers and dimers are suitable for analysis by GC (or HPLC) and GC-MS methods. Perhaps the most novel aspect of the reaction is that the primary monomeric products of this depolymerization are the lignin monomers themselves (acetylated). Another group has independently developed a related method, the TIZ method, based on similar chemistry (Nakatsubo *et al*., 1997).

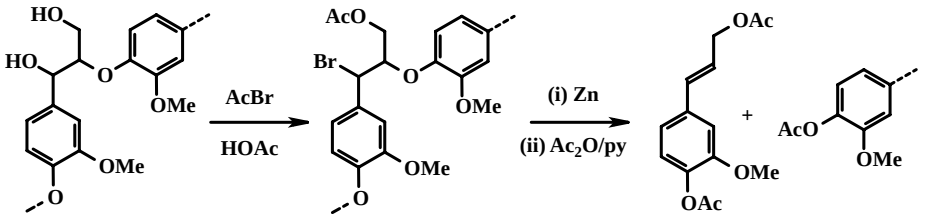


**Fig. 2. Basic DFRC Reaction on guaiacyl β-ether units.** AcBr effects cell wall solubilization while acetylating and brominating lignin units. The resulting bromo-ethers are reductively cleaved to yield hydroxycinnamyl acetates, which can be quantified by GC.

The development and application of the DFRC method has already provided useful data. For example, a new β—1-coupling pathway in lignin has been elucidated (see below) (Ralph *et al*., 1998c; Peng *et al*., 1999a), the issue of lignin optical activity could be conveniently addressed (above) (Ralph et al., 1999c), structures incorporated into lignins in plants deficient in enzymes in the lignin-biosynthetic-pathway can be nicely revealed (below) (Meyer *et al*., 1998; Marita *et al*., 1999; Peng and Ralph, 1999; Ralph *et al*., 1999a), dimers provide a wealth of structural detail (Peng *et al*., 1998; Peng *et al*., 1999b), and minor structures in lignins can be studied (Lu and Ralph, 1998a; Lu and Ralph, 1999b). Because γ-esters are not cleaved by the reactions, determining lignin acylation details has been possible (below) (Ralph and Lu, 1998; Lu and Ralph, 1999a). Chen *et al*. used the method to elegantly establish the direct biosynthetic conversion of coniferyl to sinapyl alcohol without reversion to the aldehyde (Chen *et al*., 1998; Chen *et al*., 1999).

**New Structural Features and New Mechanisms in Lignification**

Despite the maturity of lignin chemistry, there are still many puzzling aspects of NMR spectra and other data. There has been a stimulating resurgence of interest in lignin structure and some rather novel components and mechanisms have been recently unearthed. By far the most important of these is the discovery of dibenzodioxocins in lignin (Karhunen *et al*., 1995b; Karhunen *et al*., 1995a).

***Dibenzodioxocins***

5—5-Coupled units react with new monolignols to produce cyclic ether structures, dibenzodioxocins as shown in Figure 3. The previously unknown component in lignins is strikingly prevalent. Dibenzodioxocins are readily apparent in NMR spectra, particularly from 2D $^{13}C—^{1}H$-correlation (HMQC or HSQC) experiments where the

Hα—Cα and Hβ—Cβ correlations are unique. NMR was crucial to the identification of the novel units and to the unambiguous demonstration of their presence, at significant levels, in (isolated) lignins. A survey of plants reveals that dibenzodioxocins are present in all lignin classes (from softwoods and hardwoods as well as grasses and legumes, as seen here) (Galkin *et al*., 1997; Ralph *et al*., 1999b). Representative spectra from the major classes, with the dibenzodioxocins highlighted in red, are shown in Figure 4. Our group was originally concerned that such units could be artifacts of acetylation since they were difficult to find in unacetylated samples, yet readily showed up in acetylated samples. The reason appears to be that the chemical shifts are more variable in unacetylated samples, smearing out the required correlation peaks. They sharpen a little in DMSO (Ralph 1998, unpublished). Despite being ethers, such structures cannot necessarily be fully released by solvolytic methods (Karhunen *et al*., 1999).

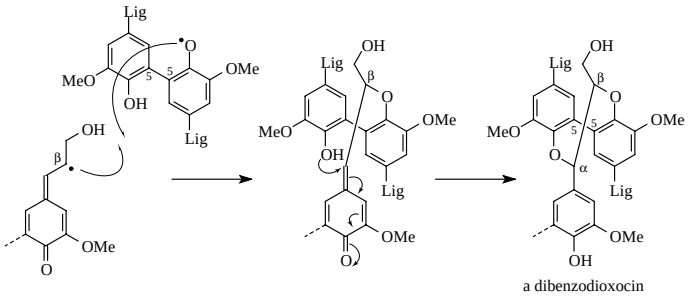


**Fig. 3. Biosynthesis of dibenzodioxocins in lignin.** Radical coupling between a 5—5-unit radical and a monolignol radical (at β) forms the β—O—4-bond as usual. Internal trapping of the quinone methide by the other phenol completes re-aromatization the process is similar to the formation of phenylcoumarans following β —5-coupling.

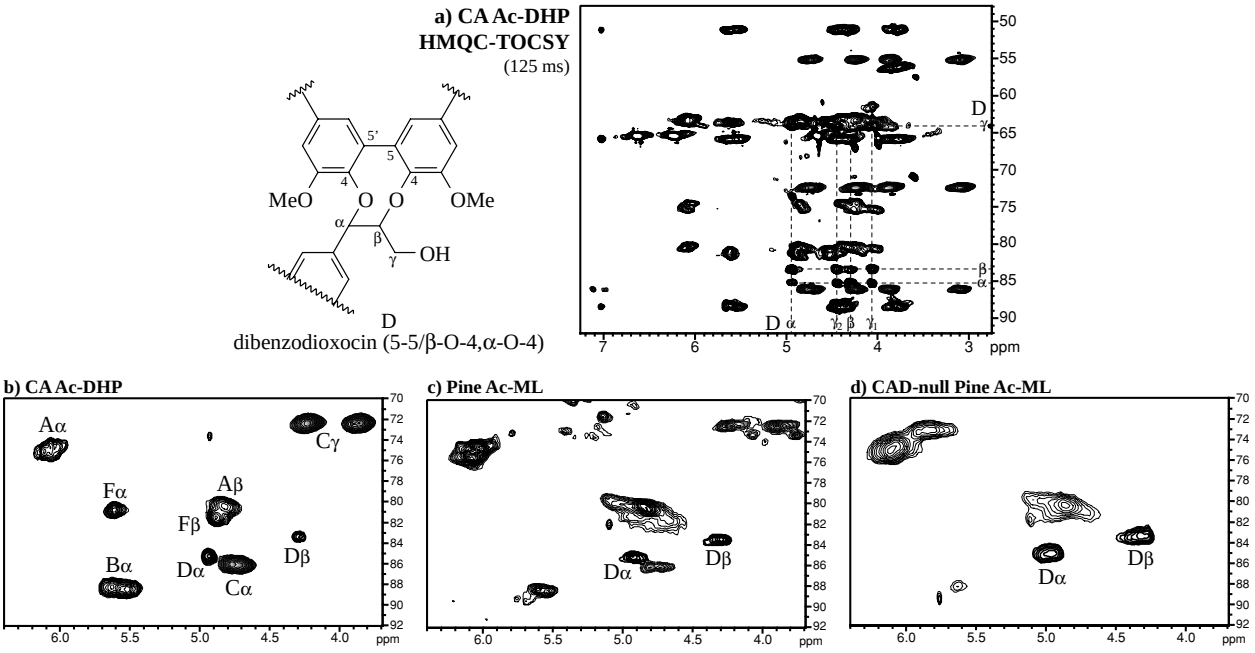


**Fig. 4. Dibenzodioxocins D are easily detected in HMQC or HSQC NMR spectra.** The Cα—Hαand Cβ—Hβ correlations are unique and readily identified. Figures b-d are HSQC spectra from: b) an acetylated DHP (derived from coniferyl alcohol in which the methoxyl group is deuterated and $^{13}$C-depleted; c) a *Pinus taeda* acetylated milled wood lignin; d) a CAD-deficient *Pinus taeda* mutant the enhanced dibenzodioxocin levels are due to the high levels of 5—5-linked DHCAunits in this lignin. Due to diverse proton-proton coupling constants, the full sidechain correlation matrix does not always show up well in HSQC-TOCSY experiments with typical (80-100 ms) TOCSY mixing times. Fig. 4a shows an HSQC-TOCSY acquired using a 125 ms TOCSY mixing time, which

beautifully emphasizes the full D coupling array. A = β-ethers, B = phenylcoumarans, C = resinols, D = dibenzodioxocin, E = α,β–diethers.

***Arylisochromans***

Recently we discovered a similar, although less prominent, product with interesting implications for the β—1 coupling pathway (Ralph et al., 1998c; Peng et al., 1999a). Aryl isochromans (**h**, Fig. 5) can be reasonably easily identified in some softwood isolated lignins by their diagnostic HMQC or HSQC correlation at $\delta_C$ 41.3, $\delta_H$ 3.60 (acetylated units, in acetone). The complete sidechain of the crucial unit is seen in TOCSY spectra. Correlations from an authenticated compound containing the isochroman trimeric structure (Ralph et al., 1998c; Peng et al., 1999a), isolated from DFRC degradation, confirm the assignment. Whether arylisochromans are present as such in native lignins is not yet clear, but even if not the internal trapping of a β—1 quinone methide intermediate (Fig. 5) is presumably operating *in vivo*. The structure differs from structures previously assigned as β—6/α–1-isochromans the regiochemistry assigned to those structures identified in hydrogenolysis and thioacidolysis products may be incorrect, i.e. they are probably β—1/α–6-compounds(Peng et al., 1999a). The rational mechanism for formation of such aryl isochromans is given in Figure 5 (Peng et al., 1999a).

Although the identification of the aryl isochroman structure in an isolated milled wood lignin can be made firmly, the apparently low amount visible in spectra does not account for the significant amounts of derived β-1 products that arise from DFRC-degradation, or various other acidolytic methods. The possibility remains that it is a product of isolation and that its precursor **d**, for example, may be the *in situ* product, as has been proposed (Brunow *et al*., 1995; Brunow, 1998). Either way, however, structure **h** provides compelling evidence for the internal cyclization pathway from β—1 intermediate **c**.

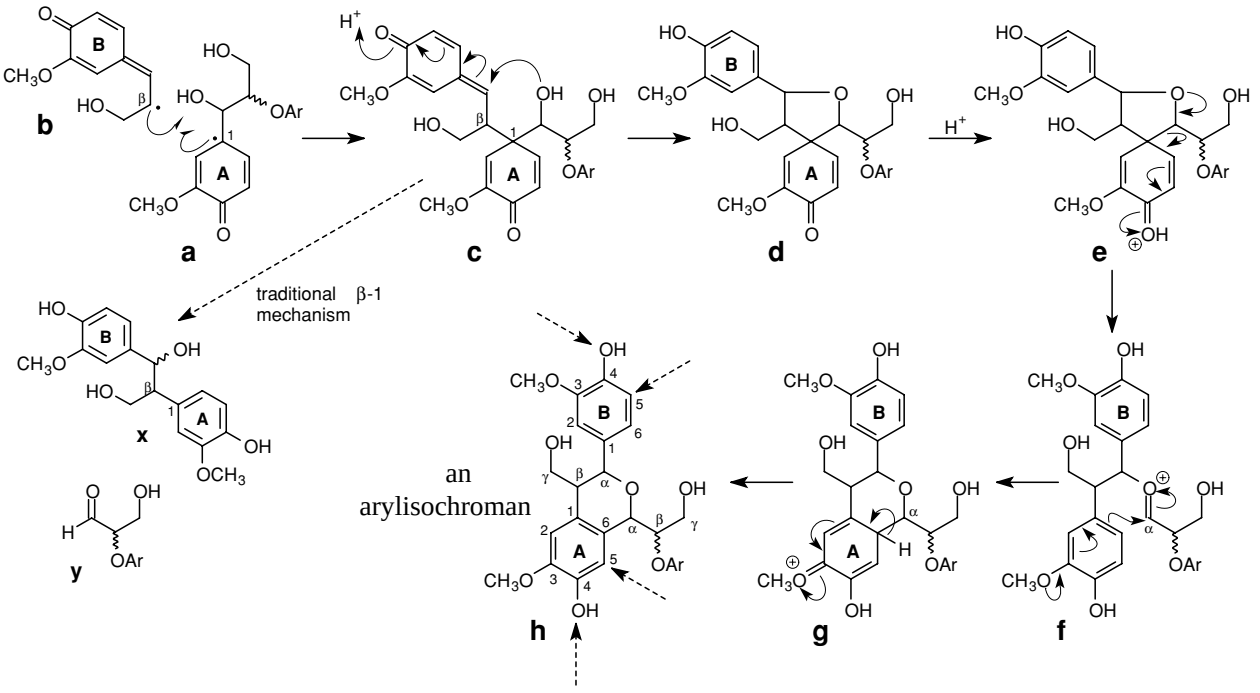


**Fig. 5. Proposed lignification mechanisms from β-1-coupling.** The sidechain of the A-unit migrates to carbon-$A_6$ eventually forming arylisochroman **h**. (Dashed arrows indicate sites for further radical coupling in lignification).

**Naturally Acylated Lignins**

As alluded to in the introduction, lignins do not arise solely from polymerization of the three monolignols. Many plants have significant quantities of other conjugates incorporated into their lignins. Acyl components include acetate, *p*-hydroxybenzoate, and *p*-coumarate.

***Acetylated lignins in Kenaf and Hardwoods***

Perhaps the most striking example to date of a highly acylated lignin is in kenaf. The bast fibers have a lignin that is well over 50% acetylated. This was established initially by NMR (Ralph, 1996). However, a general disbelief by researchers required an independent method that could be used on whole cell wall materials. A minor modification was made to the basic DFRC method all acetate-based reagents were replaced with their propionate analogues (Ralph and Lu, 1998). It is then a simple matter to differentiate fully propionylated monomers **7** (which come from normal unacetylated lignin units) from any γ-acetylated monomers **6** which must come from γ-acetylated units in the native lignin, Figure 6. The right-hand figure shows the monomeric products **6**, **7** that resulted from the modified DFRC procedure applied to isolated lignin from kenaf bast fiber; this lignin was never subjected to acetyl reagents in any form. The predominant 4-propoxy-sinapyl-γ-acetate peak **S-6** proves that syringyl units were highly acetylated in the isolated lignin. The same procedure applied to whole cell walls from kenaf bast fiber produced the central chromatogram. This sample had been extracted only with toluene:ethanol to remove extractives. Obviously, the non-isolated cell wall lignin also had syringyl units which were heavily acetylated. In both cases, only minor amounts of acetate were on guaiacyl units, **G-6**. Although the guaiacyl component of kenaf lignins is strikingly low, the preference for acetylation of syringyl components suggests a specific enzymatic process (although chemical acetylation may also be selective). Presumably this is the acetylation of sinapyl alcohol **S-1** via a transferase and activated (e.g. S—CoA) acetic acid.

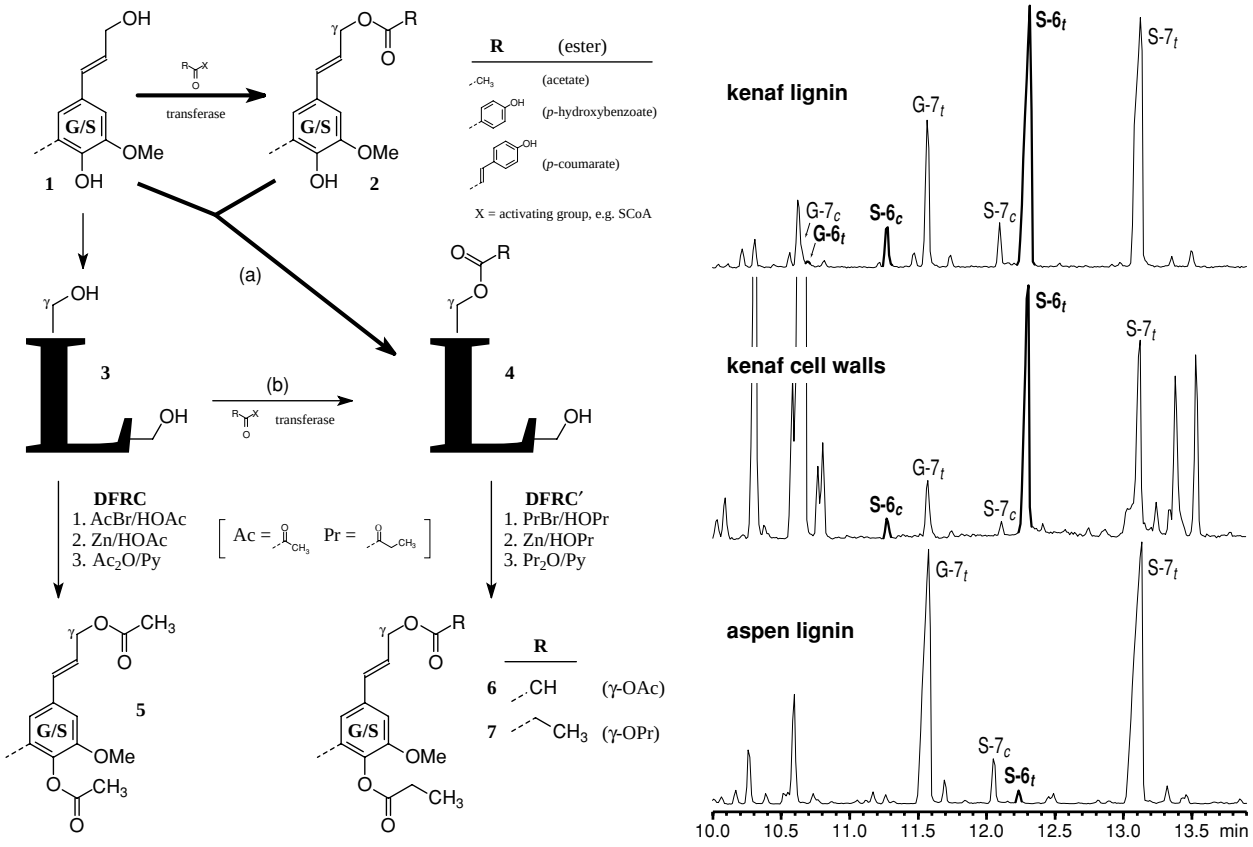


**Fig. 6. Formation and degradation pathways for acylated lignins 4.** Monolignols **1** can be acylated by suitable activated acids (acetic, *p*-hydroxybenzoic, *p*-coumaric, and others are known in nature) via transferase enzymes to monolignol esters **2**, presumably before diffusing into the cell wall. Esters **2**, along with monolignols **1**, can then be incorporated via radical coupling reactions into lignins **4** with partial γ-acylation (pathway a). Alternatively, normal lignins **3** can be subsequently acylated (pathway b). The normal DFRC protocol cleaves α- and β-ethers (in un-acylated lignins **3**) releasing, following acetylation, quantifiable monomers **5**. Lignin γ-esters (such as in **4**) are not cleaved during the normal DFRC protocol and the method can be used for identifying *p*-coumarate, *p*-

hydroxybenzoate (and presumably other) esters (not specifically shown in this figure). A modification to the DFRC protocol, substituting all acetate-based reagents with their propionate analogues (DFRC' protocol), allows determination of acetates that are naturally on lignin γ-positions. Right: GC-MS total-ion chromatograms of monomers from the modified DFRC procedure applied to a) isolated Tainung kenaf lignin, b) Tainung kenaf whole-cell walls, c) isolated aspen lignin. Components **S-6** and **G-6** (bold peaks) were from acetylated units in the original lignin; **S-7** and **G-7** were from normal (un-acylated) units. *c = cis, t = trans*. The peak labeled **S-6t** in c) contains a significant co-eluting component that was not identified. GC-MS is therefore crucial for compound authentication.

The modified DFRC procedure also allowed detection of minor acetate components in hardwood lignins (Ralph and Lu, 1998). The chromatogram of DFRC monomers from isolated aspen lignin (Fig. 6, bottom) also showed a small 4-propoxy-sinapyl-γ-acetate **S-6** peak, confirmed by MS. The analogous guaiacyl **G-6** peak could not be detected. The methods applied here do not exclude acetylation at other positions in hardwoods, but apparently, as in kenaf, a similarly selective transferase enzyme exists in aspen for acetylating sinapyl alcohol prior to its export to the wall for lignification.

***p-Coumaroylated Lignins in Grasses***

All grasses have lignins that are acylated by *p*-coumaric acid. Early attempts at establishing the acylation regiochemistry (the site on lignin where the acylation occurs) utilized UV and concluded that *p*-coumarates were mainly at the γ-position of lignin sidechains (Shimada *et al*., 1971; Nakamura and Higuchi, 1976). NMR work on corn lignin, and subsequently on wheat (Crestini and Argyropoulos, 1997), and other grasses (Ralph et al., 1999b) showed that the acylation was exclusively at the γ-position, implicating enzymatic processes in the formation of the ester. Thioacidolysis confirmed the presence of γ-acylation, although esters are partially cleaved (Grabber *et al*., 1996). The DFRC method, which leaves such γ-esters intact, further established the γ-acylation and, as for acetates, indicated that *p*-coumarates were predominantly on syringyl units (Lu and Ralph, 1999a).

***When Does Acylation Occur?***

Does the acylation occur at the monomer stage or following lignification coupling reactions (See Fig. 6)? From examination of naturally acetylated, *p*-coumaroylated, and other acylated lignins, there is reasonable, but not conclusive, evidence that acylated lignins are created from pre-acylated monomers. Perhaps the most compelling argument is that all types of units in corn are *p*-coumaroylated; this includes both β-aryl ether isomers, β—5 units, and even hydroxycinnamyl alcohol endgroups (Quideau, 1994; Ralph *et al*., 1994a). It is unlikely that the specific enzyme reaction required for this acylation would be non-specific enough to acylate such a variety of structures without also acylating α-OHs˚or even polysaccharide primary alcohols. However, unambiguous proof of monomer pre-acylation does not currently exist. Our group is currently examining an approach using the DFRC method to provide such proof.

Although not unambiguously established, it is logical that acylated lignin monomers can also couple and cross-couple with normal (unacylated) monolignols to form a lignin polymer in which some units are acylated. Thus sinapyl acetate and sinapyl *p*-coumarate are examples of possible monomers for lignification. Here we will use the term monolignol conjugate to describe these entities, since calling sinapyl *p*-coumarate a monomer can lead to confusion. The ability of lignification to incorporate monomers (or conjugates) other than the three primary monolignols is examined further below.

One initially puzzling feature of grass lignins is that the *p*-coumarate units remain free-phenolic. That is, the *p*-coumarate unit does not appear to enter into the radical-coupling lignification reactions. It is a phenol, and it is possible to make polymers from *p*-coumarate using radical-generating conditions such as peroxidase/$H_2O_2$. Furthermore, *p*-coumarates will cross-couple into a coniferyl alcohol DHP as might be expected. The reasons for its non-incorporation into natural grass lignins are now becoming clear. *p*-Coumarate˚radicals are particularly good at radical transfer reactions. Thus, although *p*-coumarate radicals are readily formed by peroxidase, they will undergo radical transfer with sinapyl alcohol or free-phenolic syringyl groups on a growing lignin oligomer regenerating *p*-coumarate and producing the sinapyl/syringyl radical (Takahama and Oniki, 1994; Hatfield *et al*., 1997; Hatfield *et al*., 1999a). This helps to explain how corn can make a syringyl-rich lignin despite having peroxidases which oxidize sinapyl alcohol only extremely poorly. Unless the system is therefore overabundant in radical producing capability (as is a normal DHP system), the *p*-coumarate is likely to remain free phenolic and uncoupled. It is suspected that *p*-hydroxybenzoates (in some grasses, notably bamboo, and some hardwoods, notably willow and aspen) may function similarly.

**Ferulate Incorporation into Grass Lignins**

A great deal is now known about ferulates in grasses (Ishii, 1997; Ralph *et al*., 1998a; Hatfield *et al*., 1999b). Dimerization of ferulate-polysaccharide esters produces a whole range of diferulates (not just the 5—5-diferulate that was the only one characterized for some 20 years) and results in polysaccharide-polysaccharide cross-linking (Ralph *et al*., 1994b). Cross-coupling of ferulates with lignin monomers (and possibly oligomers) incorporates ferulates intimately into lignin in a variety of structures (from which ferulate cannot be fully released or quantified), and results in lignin-polysaccharide cross-linking (Ralph *et al*., 1992; Ralph et al., 1998a). There is growing evidence that ferulates act as nucleation sites for lignification in grasses (Ralph *et al*., 1995). Beyond that, diferulates also incorporate into lignins via radical coupling processes forming extensive lignin-polysaccharide-polysaccharide cross-linking (Quideau and Ralph, 1997). Each of these cross-linking mechanisms negatively impacts the availability of polysaccharides for utilization by, for example, ruminant microorganisms in ruminant animals (Grabber *et al*., 1998a; Grabber *et al*., 1998c; Grabber *et al*., 1998b).

Low levels of ferulates in dicots may function in a similar way, but this has not been directly proven. Other ferulates, such as tyramine ferulate, can be incorporated into wound-response lignins, in tobacco for example (Negrel and Martin, 1984; Negrel and Jeandet, 1987; Ralph *et al*., 1998b). Tyramine ferulate is found associated with lignins in healthy tobacco plants and may therefore be a product naturally incorporated into the lignin polymer by radical coupling processes. Its content is markedly increased in cinnamoyl-Co-A deficient transgenics, although these plants have markedly reduced vigor (see below). Whether it is a true component of cell wall lignin remains to be determined.

Are ferulates (and diferulates) then to be considered lignin monomers ? Obviously ferulate is not one of the three accepted monolignols. Yet it behaves like a monomer, radically cross-coupling with monolignol radicals and fully and inextricably incorporating into the phenylpropanoid polymer. In grasses, it is likely that most lignin molecules have ferulate incorporated into them. Ferulates incorporated this way therefore analyze as lignin and there is no way to isolate or separate lignins from them. We suggest therefore that ferulates are a natural component of lignins in grasses.

**Expanding the Definition of Lignins**

It is important to note that not every entity that is attached to lignin should be considered a lignin component. The central precept of lignification is radical coupling of phenols. This concept must remain. One group has recently tried to attribute to us the absurd notion that anything that is attached to lignin should be called lignin (Kasahara *et al*., 1999). The idea that xylans, for example, are lignins just because they are attached is clearly ludicrous; xylans are not phenols and are not incorporated into lignins by radical coupling reactions. Although the concept of lignification must be expanded from the strict definition of lignins as polymers derived solely from the three hydroxycinnamyl alcohol monolignols, as recognized in the early treatises on lignification (Sarkanen and Ludwig, 1971), the core concept must remain. Lignins are also typically referred to as phenylpropanoid polymers as this is more encompassing. We would like to suggest that hydroxyphenylpropanoids, and their conjugates, that can incorporate as monomers into phenylpropanoid polymers, via radical coupling reactions might be considered lignin components. This will be addressed again below, following a discussion of lignins in lignin-biosynthetic-pathway mutants and transgenics.

**Lignins in Lignin-Biosynthetic-Pathway Mutants and Transgenics**

Researchers have recently been interested in understanding how plants respond to deviations in the normal lignification process caused by natural or biogenetic down- or up-regulation of key genes responsible for enzymes in the monolignol biosynthetic pathway. As recently stated by Stuart L. Schreiber (Anonymous, 1999); If you want to understand something it s very useful if you can perturb it. Complex biological systems can be thought of as complex circuitry. You need ways to break circuits, turn them on, turn them off, modulate them, in order to understand them. As we are learning, however, the lignification system has considerable metabolic plasticity. Breaking circuits does not necessarily turn off the process.

The potential to improve plant utilization by ruminants and in various other natural and industrial processes (such as chemical pulping) by engineering the amount, composition, and structure of lignin is currently attracting considerable interest. The major approach along these lines is to target enzymes of the monolignol biosynthetic pathway, as recently reviewed (Boudet *et al*., 1993; Halpin *et al*., 1994; Boudet and Grima-Pettenati, 1996; Boudet, 1998; Whetten et al. 1998). Various plants with deficiencies in lignin-biosynthetic-pathway genes (and consequent

enzymes for monolignol synthesis) provide insights into the flexibility of the lignification process and aid in our understanding of normal lignification.

***Aspen Pt-4CL Transgenics.*** Chiang s group have generated transgenic aspen with suppressed expression of 4-coumaric acid:coenzyme-A ligase (Pt4CL1) (Tsai *et al*., 1994; Hu *et al*., 1998). The enzyme functions early in the monolignol pathway and the researchers reasoned that this was a good target for effecting general lignin down-regulation. The downregulation of lignin was not only striking (up to a 45% reduction), but was also accompanied by a 15% increase in cellulose the cellulose:lignin ratio nearly doubled (Hu *et al*., 1999). The benefits of vigorous plants with these properties for pulp and paper production and in forages are obvious.

Milled wood lignins from a control (42% of the total Klason lignin) and the most heavily down-regulated transgenic (28% of the total original Klason lignin) were examined by NMR (Hu et al., 1999). The lignin in the transgenic was not significantly different from that in the control, validating the choice of this strategy for effecting a general reduction in the amount of lignins in plants (without massively affecting the composition). The plant appears to be supplanting its reduced lignin with additional cellulose. If the required defense, water transport, and other properties survive, as they appear to in these particularly vigorous plants, the potential for improving plant utilization is enormous.

***Arabidopsis F5H-Mutants and Transgenics.*** An illustration of the massive compositional changes that plants can tolerate is strikingly revealed in mutants and transgenics of ferulate 5-hydroxylase (F5H) from Chapple s group (Meyer et al., 1998; Marita et al., 1999; Ralph et al., 1999b). Ferulate 5-hydroxylase (enzyme F5H) is the first enzyme that controls the production of sinapyl alcohol and therefore syringyl units (Meyer *et al*., 1996; Meyer *et al*., 1997; Chapple, 1998; Meyer et al., 1998). An F5H-deficient mutant, produced by chemical mutagenesis, is almost totally devoid of syringyl components, producing a guaiacyl lignin that would be typical of a softwood (rather than the dicot it is). More impressively, upregulating F5H in the mutant produces transgenics that are almost 100% syringyl (and devoid of guaiacyl units). Thus the lignin composition can be altered from essentially 100%G/0%S to 0%G/100%S in a single generation, without the benefit of evolutionary adaptation. Both lignins are otherwise normal. The syringyl content of these transgenics is significantly higher (by a factor of 4!) than in any plant reported to date (Marita et al., 1999; Ralph et al., 1999b). The up-regulation is therefore strikingly effective at diverting the monolignol pool into almost entirely sinapyl alcohol. This example is one in which massive compositional shifts have been effected, but in the traditional lignin components. As will be seen, compositional shifts can range considerably wider.

***Pine CAD-deficient Mutant.*** A CAD-deficient pine mutant was identified and characterized by workers at North Carolina State University (MacKay, 1997; MacKay *et al*., 1997). The pine, despite a reduction in CAD levels to less than 1% of normal levels, appeared to be producing normal levels of lignin (Klason lignin values were almost identical). A possible interpretation then is that the CAD-deficiency is not sufficient to change the system and that there is still sufficient flux through the residual enzyme levels that normal lignification can proceed. The lignin in the CAD-deficient pine mutant however was highly colored and stained strongly with phloroglucinol, suggesting a higher aldehyde component. From NMR studies on isolated lignins, it was clear that aldehydes were substantial components of that lignin (Ralph *et al*., 1997). New aldehyde components were misidentified originally (Ralph et al., 1997), but corrected in subsequent publications (Ralph et al., 1998b; Ralph et al., 1999b). A book chapter on NMR even details how the incorrect assignment was made (Ralph et al., 1999b). The aldehyde components now clearly all stem from incorporation into the lignin of more significant quantities of coniferaldehyde (and its product vanillin) than in normal pine. Is this aldehyde component part of the lignin? The low levels seen in normal lignins could easily result from post-lignification oxidation, but the levels seen in this and other CAD-deficient plants (see tobacco below) suggest that incorporation of hydroxycinnamaldehydes (and hydroxybenzaldehydes) into lignins is a normal process that becomes more significant when the levels of monolignols are insufficient for lignification.

Even more striking, however, was the observation (Ralph et al., 1997) of substantial levels of dihydroconiferyl alcohol (DHCA) in the mutant s lignin. Lewis claimed that DHCA was not a monomer and only resulted from post coupling ancillary lignan reactions (Gang *et al*., 1998; Lewis *et al*., 1998). Our evidence that DHCA had to be a monomer in the production of the phenolic polymer we isolated was several-fold. Firstly, DHCA monomer was released in considerable amounts in extracts from the wood of the mutant pine. Secondly, degradation by our DFRC method (Derivatization Followed by Reductive Cleavage) produced significant quantities of DHCA proving that they were etherified. Even though they are likely to more normally couple in ways that cannot release the monomer (5—5 and 5—O—4 or 4—O—5) the amount released was still comparable to the (lower level of) coniferyl alcohol that was released. And the amounts released were significant whether the sample was the isolated lignin, the lignin

residue following isolation, or the whole wood. These observations have recently been independently borne out by thioacidolysis (Lapierre *et al*., 1999). Thirdly, and far more compellingly, NMR showed that about half of the DHCA units were involved in 5—5-coupled structures (Ralph, 1997; Ralph et al., 1997.)As anyone who has studied dimerization of coniferyl alcohol knows, the coupling almost entirely involves at least one coniferyl alcohol coupling at its β-position; the observed dimers are the β–β- (pinoresinol), the β—5- (phenylcoumaran), and the β—O—4-dimers. Under no conditions reported to date have 5—5-coupled dimers been reported as substantial products. How then can 50% of the DHCA units be 5—5-coupled if they arise post-dimerization? Obviously they do not. The logical conclusion is that it is DHCA monomer that is undergoing the radical coupling reactions. As we will show below, there is now even more evidence for this assertion. It is also logical, since DHCA is known to prefer coupling reactions with radicals of its own kind (rather than coniferyl alcohol radicals), that much of the 5—5-DHCA units likely arise from such homo-coupling reactions when the levels of DHCA are so significant. It should be noted that evidence for DHCA units has been provided in numerous normal lignins (Lundquist and Stern, 1989; Fukagawa *et al*., 1991; Brunow *et al*., 1998; Ralph et al., 1999b).

GC-MS of DFRC dimers (Peng et al., 1998) from the mutant vs a normal pine reinforces the striking differences, Figure 7. The dimers, like thioacidolysis dimers, don t represent a huge fraction of the lignin, just those units that can be released by ether-cleavage reactions. But they can be used as diagnostic fingerprints of the polymer structure. In the normal lignin, we can identify and recognize anticipated dimers from a softwood lignin. These dimers are present in small amounts in the mutant s product, but are overwhelmed by a series of new dimers. From the fingerprint alone, it is obvious that these two lignins must differ greatly. The new dimers arise from DHCA (and DHCA-derived) units and aldehyde units in various combinations. Incidentally, the same products in similar distributions arise when whole-cell-wall samples, rather than simply the isolated lignins, are subjected to DFRC.

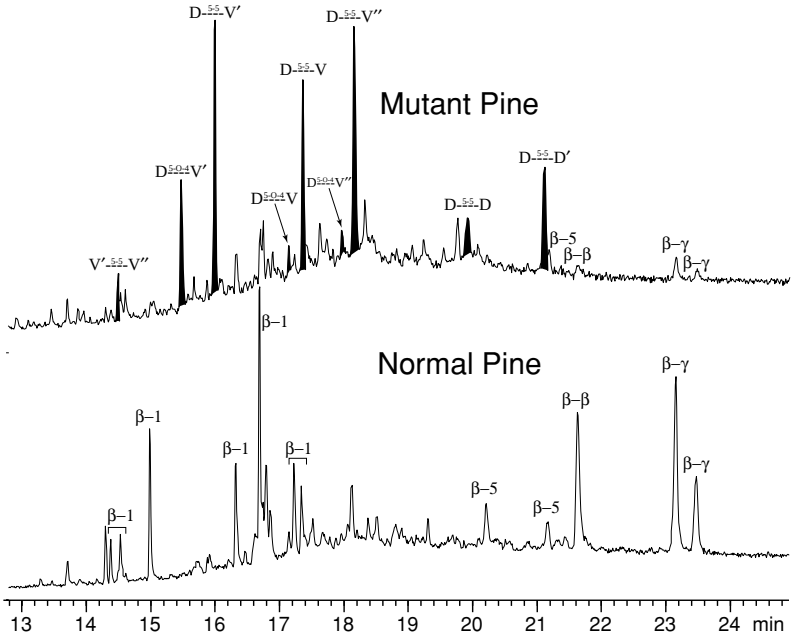


**Fig. 7. DFRC dimers from normal and mutant pines.** DFRC dimers from normal vs mutant pine lignins. Although the normal dimers can be detected in the CAD-deficient mutant, they are overwhelmed by new products that are insignificant in normal pine. These new dimers are all various combinations of DHCA (D) (or its derived guaiacylpropane-1,3-diol (D')) and vanillin (V) or its DFRC-derived products (V', V''). The same products appear in similar distributions whether the DFRC-degradation is performed on the isolated lignins or whole-cell-wall materials. From the fingerprint alone, it is obvious that the two lignins are significantly different.

***Newly identified components of the mutant pine s lignin.*** Recently we have been able to identify another major new component in the lignin of the mutant pine. Again, this new component is seen at trace levels in the normal pine. The units are arylpropane-1,3-diols, Fig. 8. At first it is difficult to understand how these can arise, but

in fact they stem directly from peroxidase reactions of DHCA. Figure 8 shows the NMR proof of the presence of these structures in the mutant s isolated lignin. This new unit provides additional proof that DHCA is a monomeric component of the phenolic polymer. Some of the new peaks in the DFRC dimer fraction from the mutant are attributable to coupling products of this new arylpropane-1,3-diol unit with DHCA and aldehyde units. Finding the oxidized α-keto-product (in collaboration with Jay Scott, NC State) provided further validation of the production of arylpropane-1,3-diols.

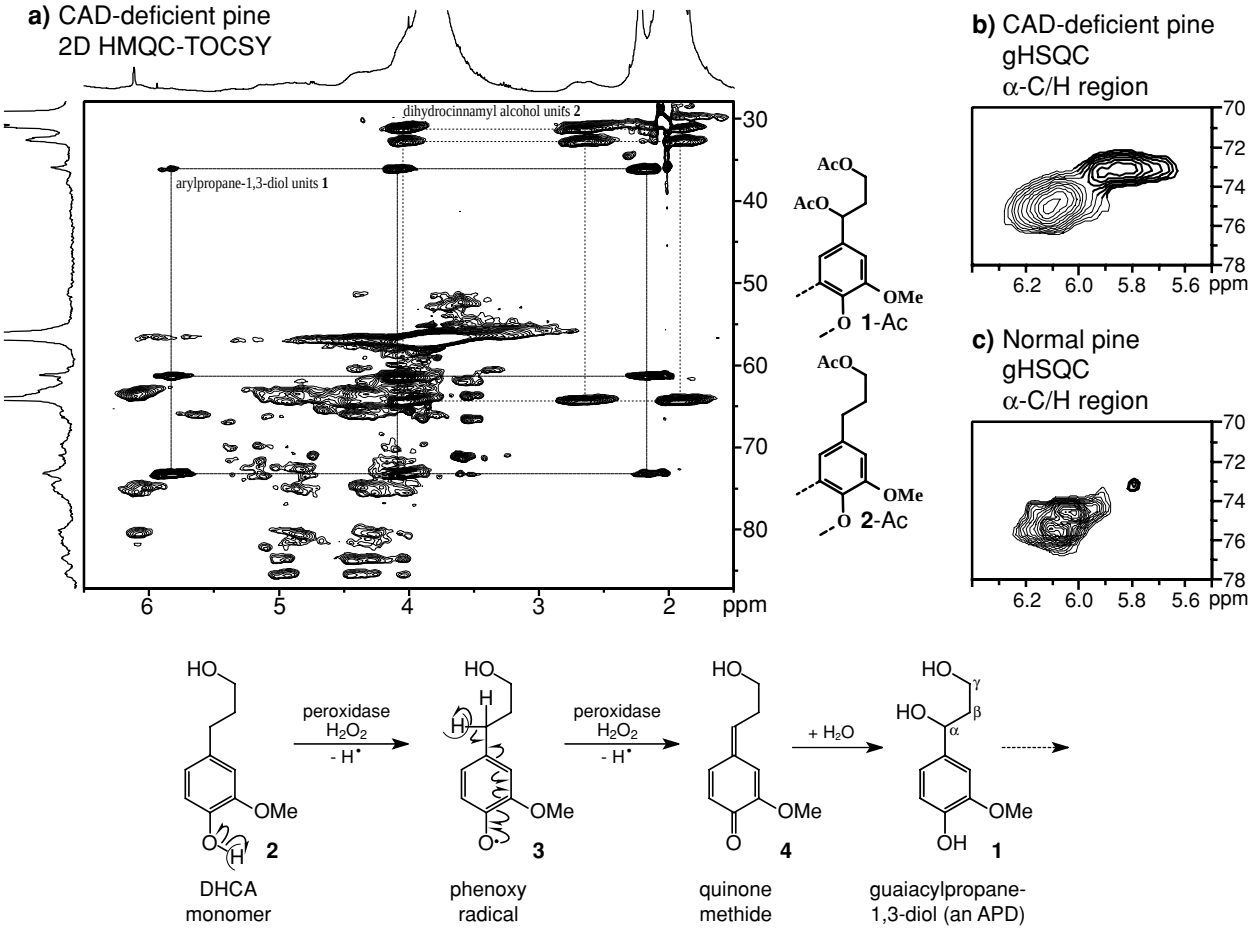


**Fig. 8. NMR spectra showing new APD structures.** Left: HMQC-TOCSY showing APDs (bold), along with DHCA units previously identified. Figs. **b-c** are expansions from HSQC spectra showing the APD units Hα/Cα correlations in normal and mutant pine.

***Is this polymer lignin??*** The mutant and the normal pine have similar lignin levels as measured by Klason analysis. If lignins cannot, by definition, contain substantial portions of DHCA, DHCA-derived products, and aldehydes, then the mutant obviously has very little, if any, lignin. That is great news! It is one of the prime objectives of biogenetic engineering work in this area to reduce the level of lignin. But if the lignin reduction comes *only* because of semantics, because of the definition of lignin, and not by a real reduction in the phenolic polymer component (that may be functioning as lignin in the plant!), then obviously no real benefits (for pulping, for example) have been gained. The question of whether the plant utilizes this polymer as lignin awaits further debate and experimentation. At this point, since these DHCA and aldehyde components appear to be likely components of normal lignins, we think this is merely another example of a broad compositional shift.

***Tobacco CAD- and CCR-Down-regulated Transgenics***. Tobacco CAD-deficient transgenics were produced in Alain Boudet s group (Piquemal *et al.*, 1998; Yahiaoui *et al.*, 1998). Again, the lignin levels in a CAD-

downregulated transgenic were similar to the control, and fibers had the brown coloration. And again, the CAD-down-regulated transgenics showed a significant buildup of aldehyde components. New products due to homo- and hetero-coupling reactions of coniferaldehyde/sinapaldehyde are prevalent and are analogous to the coniferaldehyde products seen in the CAD-deficient pine mutant. No DHCA units were found, but there may be no pathway for their upregulation DHCA is seen less commonly in NMR spectra of lignins of plant materials other than softwoods, and is not at detectable levels in any of the tobacco isolated lignins.

Transgenics with CCR (cinnamoyl-CoA reductase) downregulated (Piquemal *et al*., 1998) by antisense methods had significantly lower (~50%) lignin contents in plants that were clearly less vigorous. The isolated lignin showed a striking increase in tyramine ferulate, which was shown to be incorporated in as an integral component of the polymer (Ralph *et al*., 1998b). Tyramine ferulate is a logical sink-product for feruloyl-CoA when CCR is down-regulated in tobacco. However, it is a normal wounding response product and there are again issues of whether this component is truly incorporated into structural lignin, into suberized components (Bernards and Lewis, 1998), or simply represents polymerized secondary metabolites similar to those observed in a wounding response. However, the aliphatic components normally associated with suberins are not significant in these extracted lignins. Also, tyramine components are not elevated in the antisense-CAD tobacco which was presumably also stressed. The dilemma of deciding whether tyramine ferulate is therefore a lignin component or not remains. (Is wound-response lignin still considered lignin?).

***OMT-Deficient Plants.*** Naturally occurring mutants (*e.g*., the brown-midrib (bm3) mutants of maize and sorghum and transgenic plants deficient in *O*-methyl transferase (OMT) contain significant amounts of units derived from 5-hydroxyconiferyl alcohol, as revealed in beautiful thioacidolysis studies (Lapierre *et al*., 1988; Lapierre, 1993; Atanassova *et al*., 1995; Doorsselaere *et al*., 1995). We have not yet looked at such plants (by NMR) but it seems logical that the unmethylated precursors to sinapaldehyde could be incorporated into lignins if they were to build up in the system due to reduced OMT activity. Is it also reasonable that 3,4-dihydroxycinnamyl alcohol could similarly incorporate.

**Components in Lignins, Summary**

There are many known lignins that are derived from incorporation of precursors or derivatives of the three monolignols, i.e. from components other than the monolignols themselves. The idea that other components are incorporated into lignins is nothing new. If they are truly foreign components from other pathways that are not laid down during lignification, then they should be distinguished. But where the polymer contains intimately incorporated components that can only arise from radical coupling reactions, particularly involving monomers, then nature may be defining them as lignins even though some of us can not. Many lignins, for example, are biosynthesized by incorporating esterified monolignols into the lignification scheme. As detailed above, grasses utilize *p*-coumarates, hardwoods and some dicots such as kenaf utilize acetates, and some plants, notably bamboo, aspen and willow, use *p*-hydroxybenzoates as monomers for lignification. Ferulates and diferulates are found intimately incorporated into all grass and some dicot lignins, where they are equal partners in the free-radical polymerization process and may even be nucleation sites for lignification. If aldehydes, tyramine hydroxycinnamates, dihydro-monolignols, 5-hydroxyconiferyl alcohol, and other precursors from the studies on mutants and transgenics are considered, it becomes clear that a general definition of lignin must include more than the traditional three hydroxycinnamyl alcohols, Fig. 9. If not, the phenolic polymers serving the structural and functional role of lignin in many plants, *e.g.*, in grasses, might not be considered lignin.

**Conclusions**

Lignin remains a complex enigma, and structural details of the phenylpropanoid polymers in plants are still emerging. Applications of new and traditional methods continue to reveal a fascinating complexity. The number of monomers or monomer conjugates that undergo radical coupling reactions with typical monolignols to generate complex phenylpropanoid polymers continues to grow. Whether these are true lignins or not continues to be debated. However, components that act as monomers, coupling and cross-coupling with monolignols and oligolignols to become intimately incorporated into the structure are obviously important components of the plant s architecture. These components will always analyze as lignin and for some industries such as the pulp and paper industry, will remain the troublesome polymeric phenolic components that must be removed during pulping or bleaching to produce high-end products such as white paper. Structural analysis of normal and perturbed plant systems reveals a lot about the phenylpropanoid component and interacting biochemical pathways, providing a basis for in-depth biochemical studies. Structural elucidation remains a key entry point into understanding these complex

plant systems. Instrumental and degradative methods continue to evolve to provide increasingly sophisticated probes to detail structural and mechanistic aspects of lignification.

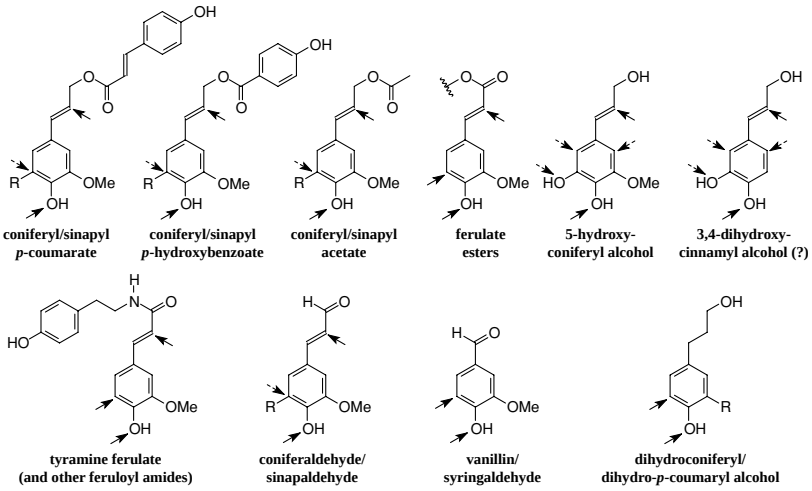


**Fig. 9. Compounds which function like monomers** in free-radical coupling reactions that typify lignification. Arrows show sites available for radical coupling.


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